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The Heterogeneity of Human Chorionic Gonadotropin (hCG). I. Characterization of Peptide Heterogeneity in 13 Individual Preparations of hCG*

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ABSTRACT. Peptide variations in the α-subunit (molecules starting at α3 and α4) and β-subunit (missing linkages at β44–45 and β47–48) of hCG have been reported by several investigators. Studies, however, have been limited to standard hCG preparations (purified from large pools of urine) and other hCG samples from mixed urines. In this study we used chromatographic procedures to purify the total hCG content of 13 individual urines, 6 from patients with pregnancy and 7 from those with trophoblast disease (no hCG-containing fractions were excluded). Then, we examined for the first time the peptide variability among individual samples of hCG. We report 1) that individual hCG preparations have nicks (missing linkages) in the β-subunit, primarily between residue 47–48 (11 of 13 samples) and, less commonly, at the linkage 44–45 or 46–47 (3 of 13 samples); 2) the extent of nicking varies greatly between individual preparations (range, 0–100% of molecules); 3) varying α-subunit N-terminal heterogeneity (N-terminus starting at α3 or α4) was also present (range, 0–28% of molecules), but was confined to preparations from individuals with trophoblast disease (6 of 7 samples from trophoblast disease urine, 0 of 6 from pregnancy urine); 4) hCG missing the β-subunit C-terminal region was also detected (2 of 13 hCG preparations); and 5) 1 of 13 preparations was nicked on the hCG α-subunit, between residues 70 and 71. Thus, 12 of 13 individual hCG samples demonstrated at least 1 of 4 different forms of peptide heterogeneity. We conclude that individual hCG samples vary widely in the type and extent of peptide heterogeneity, an observation that is not appreciated when pools of hCG are studied. (*Endocrinology* 129: 1541–1550, 1991)

CG IS a glycoprotein hormone produced by the placenta in individuals with pregnancy and trophoblast isease (hydatidiform mole and choriocarcinoma). hCG onsists of two dissimilar subunits, α and β, joined nonovalently (1, 2). The three related hormones, human (h) H, hFSH, and hTSH, share a common α-subunit with CG. Each, however, has a unique β-subunit, giving them eir individual functions (3). The α-subunit of hCG onsists of 92 amino acids with *N*-asparagine-linked igosaccharides at residues 52 and 78. The β-subunit of CG consists of 145 amino acids. Like the α-subunit, it as *N*-asparagine-linked oligosaccharides (residues 13 nd 30), but has, in addition, α-serine-linked sugar structres in the C-terminal segment of the molecule at residues 121, 127, 132, and 138 (1, 2, 4, 5).

The α- and β-subunits are found in blood and urine, oth associated as hCG dimer and uncombined as free α- and β-subunits (6–8). A related molecule, β-subunit core fragment, which comprises β-subunit residues 6–40 disulfide linked to residues 55–92, is also found (8–10). Using isoelectric focussing, electrophoresis, and related methods, hCG microheterogeneity has been demonstrated (for reviews, see Ref. 11). This heterogeneity and other small differences in the size and charge of hCG have been attributed primarily to variations in the sialic acid content and oligosaccharide structures of the carbohydrate moieties. Heterogeneity has also been observed in the N-terminal amino acid sequence of the α-subunit (NH_2Ala-Pro-Asp-Val-Gln-), with as many as 20% of the molecules starting at α3 (NH_2Asp-Val-Gln-) or α4 (NH_2Val-Gln-) (12). Furthermore, nicks or missing peptide linkages have been reported in the hCG β-subunit between residues 44 and 45 (-Val-Leu-) or between 47 and 48 (-Gly-Val-) in a varying proportion of molecules (8, 12–16). The findings of peptide heterogeneity, however, are based on sequence analyses that were performed on hCG purified from large pools of urine, either standard hCG (from Organon crude) or other urine mixtures (15, 16). These results from sequences of standard or pooled urine hCG may not be representative of those

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from individual urine samples. We report here the purification of a significant number of individual hCG samples (n = 13) and, for the first time, the variation in peptide structure in individual hCG preparations.

In the companion papers peptide variations in standard hCG preparations are examined, the origin of β-subunit nicking is studied (17), and the effect of nicking on hCG biological and immunological activity is investigated (18).

Materials and Methods

Samples and purification of hCG

Thirteen individuals collected 24-h urine samples. All urine samples were kept frozen during the collection period. As summarized in Table 1, six of the urine collections were from pregnant women, three from patients with hydatidiform mole, and four from those with choriocarcinoma or invasive trophoblast disease. The volume of urine collected ranged from 0.4–10 liters. hCG was purified from urine by a modification of the extraction procedures of Van Hell *et al.* (19) (ethanol precipitation) and the chromatography methods of Canfield and Morgan (20) (DEAE ion exchange, followed by gel filtration). Figure 1 is a flow diagram detailing the purification methods used in this study. As shown in Fig. 1, an additional extraction step, using acetone precipitation before ethanol precipitation, was added. This was included to optimize removal of lipids and hydrophobic micromolecular contaminants. In the gel filtration and ion exchange chromatography steps, fractions were assayed by Hybritech Tandem assay. To meet our objective of determining the peptide heterogeneity of hCG, no form of hCG could be excluded. Therefore, chromatography fractions were collected from the peak of hCG immunoreactivity to a level of 1% of peak levels on either side. A consequence of this approach was that the two chromatography steps were not sufficient to purify the hCG. Therefore, an extra step, high resolution gel filtration, was added to optimize the purification. In the instances when a significant second hCG peak was detected in

TABLE 1. Collection of pregnancy and trophoblast disease patient urine

Urine donors	Time urine collected; clinical observations	Vol collected (liters)	Total hCG (mg)
Pregnant			
P1	8 weeks since last period; normal singleton pregnancy	0.4	6.0
P2	5.5 weeks since last period; normal singleton pregnancy	4.0	1.8
P3	9 weeks since last period; normal singleton pregnancy	8.0	11
P4	16 weeks since last period; normal singleton pregnancy	6.4	1.8
P5	8 weeks since last period; normal singleton pregnancy	2.5	3.4
P6	7.5 weeks since last period; outcome unknown	3.5	3.5
Those with hydatidiform mole			
M1	0 and 1 day postevacuation; pathology: complete mole	2.6	15
M2	Preevacuation; pathology: complete mole	1.8	11
M4	Preevacuation; pathology: complete mole	5.2	110
Those with choriocarcinoma or invasive trophoblast disease			
C1	Pretherapy; lung and brain metastases	1.0	12
C2	During resistance to therapy; lung and brain metastases	4.9	15
C3	During resistance to therapy; lung metastases	10	7.8
C5	Pretherapy; lung metastases	2.0	85

Urines were collected by Laurence Cole or Ernest Kohorn at Yale University (P1-P5, M1, and M4), and by Laurence Cole at University of Michigan (P6), Gunma University in Japan (C3), Charing Cross Hospital in London, England (C2), and Liaoning Tumor Institute in People's Republic of China (C5). Further urine samples were collected by Jerome Belinson at University of Vermont (M2), and by Mehmet Ozturk at Harvard University (C1). All urine samples were frozen immediately after collection.

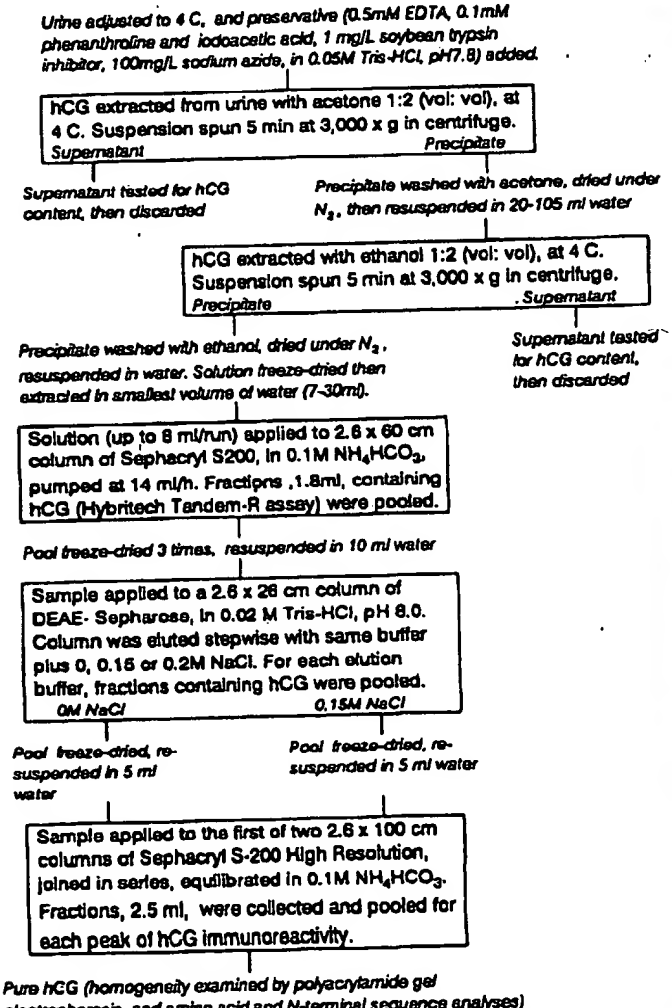

FIG. 1. Scheme for the purification of hCG from urine samples of 13 individuals with pregnancy or trophoblast disease.

e earlier chromatography step (DEAE-Sepharose), the ma-rial was pooled and purified separately.

'ectrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis as carried out on 12% or 16% polyacrylamide gels in a Bio-ad Mini Protean II slab gel apparatus (Richmond, CA), using ıe discontinuous buffer system of Laemmli (21). Samples were ıixed with an equal volume of reducing buffer [0.5 M Tris-[Cl, pH 6.8, with 5% glycerol, 1% sodium dodecyl sulfate, and .5% (vol/vol) β-mercaptoethanol], heated to 80 C for 10 min, ɔoled, then applied to gels. The gels were subjected to 200 V ɔr 1 h at ambient temperature. Gels were either silver stained ɔr protein, using previously described methods (9), or blotted ɔr immunostaining. Blotting was carried out in a Bio-Rad Mini Trans-Blot apparatus, using the methods of Burnette 22). Briefly, gels were placed on a 0.2-μm nitrocellulose mem-ɔrane, fitted into the transfer apparatus, and subjected to 100 / for 1 h at ambient temperature. Proteins transferred onto :he membrane were washed free of sodium dodecyl sulfate, and :he membranes were blocked from any further nonspecific ɔinding by soaking in PBS (pH 7.5) containing 5% BSA. Nitrocellulose membranes were then incubated for 4 h at ambient temperature with antiserum raised to hCG β-subunit C-terminal peptide (residues 130–145, CC11, kindly donated by Dr. H. C. Chen of NIH), and bands were visualized by further incubation with protein-A-gold conjugate (Bio-Rad). Added sensitivity was achieved by a gold enhancement procedure (no. 170–6538, Bio-Rad). This consisted of soaking the washed membranes with 0.2 M citrate buffer (pH 3.7) and then incubating with a solution of 0.85% hydroquinone plus 0.11% silver lactate for 15 min in the dark. The reaction was stopped using the suppli d fixing solution. Nitrocellulose membranes were scanned on a Hewlett-Packard 300 × 300-dpi scanner (Palo Alto, CA), using Scangal-5 software with 256 Gray scales, and printed on a 300 × 300-dpi laser printer.

Immunoassays

hCG levels were determined using a two-step modification of the Tandem kit from Hybritech (San Diego, CA), as previously described (23). This assay uses an α-subunit monoclonal antibody to capture hCG and a β-subunit monoclonal to label bound hormone. Briefly, 100 μl sample plus 200 μl PBS-O (PBS, pH 7.5, with 0.1% ovalbumin) were incubated for 2 h at ambient temperature with anti-α-subunit monoclonal antibody bound to a glass bead. After washing the bead with water, 100 μl ^{125}I-labeled anti-β-subunit monoclonal antibody plus 200 μl PBS-O were added, and incubation was continued for a further 2 h at ambient temperature. After washing the bead with water, radioactivity was determined. This assay is specific for hCG, with less than 0.1% cross-reactivity with hCG free β-subunit, free α-subunit, or hLH. Values were determined in triplicate and expressed as micrograms per ml, using hCG batch CR127 (NIDDK) as standard.

The presence of the C-terminal peptide on hCG was determined using a specific immunoradiometric assay. The method used was a modification of the sandwich procedure (anti-β-antiserum:anti-β C-terminal segment antiserum) of Armstrong and colleagues (24), using the ^{125}I-labeled anti-β monoclonal antibody from the Hybritech Tandem kit in place of anti-β antiserum. Procedures were the same as those described above for the Hybritech Tandem assay, except that 100 μl of a 1:5 suspension of C-terminal peptide antiserum-Sepharose 4B were used as a substitute for the bead. On a molar basis, this assay equally recognizes hCG and its free β-subunit. The cross-reactivity with hLH and with hCG or β-subunit missing the COOH-terminal segment was zero.

Free β-subunit was measured using monoclonal antibody FBT 11 (a gift from Dr. J.-M. Bidart, Institut Gustave-Roussy, Villejuif, France). Microtiter plates were incubated overnight at 4 C with 200 μl 0.5 μg/ml antibody FBT 11 in 0.25 M sodium bicarbonate-0.1 M sodium chloride buffer, pH 9.5. After washing, 100 μl sample or standard (CH129 hCGβ, NIADD) and 100 μl PBS-O were added, and plates were incubated with shaking for 1 h at ambient temperature. The plates were again washed, 200 μl goat anti-hCGβ-peroxidase (Bios Pacific, Inc., Emeryville, CA) were added, and incubation was continued for a further 2 h at ambient temperature. Color was developed using *ortho*-phenylene diamine, and the absorbance was measured at 492 nm on a Titertek Multiskan MCC/340® (Flow Laboratories, Inglewood, CA) plate reader. This free β-subunit assay had 2.5% cross-reactivity with intact hCG.

Amino acid sequence analysis

Sequence analysis was performed on an Applied Biosystems model 470A gas phase sequencer (Applied Biosystems, Foster City, CA) equipped with an on-line phenylthiohydantoin analyzer. Peaks were quantified using Nelson Analytical Xtrachrom software.

Amino acid analysis

Protein samples were hydrolyzed with 6 M HCl at 110 C for 24 h. Analyses were performed on a Beckman 6300A automated amino acid analyzer (Somerset, NJ).

Carboxypeptidase-Y digestion

hCG preparation C5 (100% nicked at β47–48) was reduc d and alkylated, and peptides (α- and β-subunit N- and C-terminal segments) were separated by reverse phase HPLC [methods described in the following paper (17)]. The β-subunit N-terminal peptide was used for C-terminal sequence studies. Three nanomoles of peptide and norleucine (internal standard, Pierce, Rockford, IL) were dissolved in 180 μl 0.05 M sodium acetate, pH 5.5. A zero time point sample was prepared by transferring 40 μl peptide solution to a tube containing 0.4 μg carboxypeptidase-Y (Boeringer Mannheim, Indianapolis, IN) and 5 μl trifluoroacetic acid. To the sample to be digested, 1 μg carboxypeptidase-Y was added, and the solution was incubated at 37 C. Aliquots of 40 μl were taken at 1, 3, and 10 min and transferred to tubes containing 5 μl trifluoroacetic acid. A blank containing only norleucine and enzyme was treated in the same way. All aliquots were dried and analyzed for free amino acids on an Applied Biosystems 420A analyzer (Foster City, CA).

Results

We purified hCG from urine from six women with normal pregnancies, three women with hydatidiform

mole, and four women with choriocarcinoma (Tables 1 and 2), using a five-step organic extraction-chromatography procedure (Figs. 1-3). During the purification, emphasis was placed on collecting all hCG-immunoreactive material. In the chromatography steps, pools of fractions included all those that contained more than 1% of peak hCG immunoreactivity. Figure 2 shows examples of elution profiles from the first two chromatography steps (initial gel filtration and ion exchange) and indicates the fractions pooled. Figure 3 shows examples of elution profiles for the final chromatography step (high resolution gel filtration) and indicates the fractions pooled. The final recoveries after the five purification steps were 38%, 31%, 38%, 44%, 49%, and 51%, respectively, for samples P1-P6; 49%, 38%, and 40% for M1, M2, and M4; and 32%, 37%, and 57% for C1, C3, and C5. Losses at each step were similar for each sample. Nonparallel results in the immunoassay of C2 starting urine precluded determination of its recovery.

The hCG preparations (main peaks) were examined after reduction, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4). In 12 of the 13 samples, bands were only detected in the position of hCG α-subunit [mol wt (M_r), 22,000–24,000), hCG β-subunit (M_r, 34,000–37,000), or β-subunit N-terminal fragment (β1–47; M_r, 17,000) (8, 15). The latter was only observed in nicked hCG preparations (peptide β48–145 comigrates with hCG α-subunit). The exception, sample C2, had an additional band (M_r, 50,000).

TABLE 2. DEAE-Sepharose ion exchange chromatography of partially purified hCG preparations

Sample code	hCG immunoreactivity (μg eluted)		
	0.02 M Tris-HCl, pH 8	0.02 M Tris-HCl, pH 8 + 0.15 M NaCl	0.02 M Tris-HCl, pH 8, + 0.20 M NaCl
P1	0 (<1)	3,800 (>99)	5 (<1)
P2	15 (2.6)	560 (97)	5 (<1)
P3	1 (<1)	3,600 (>99)	19 (<1)
P4	0 (<1)	990 (>99)	0 (<1)
P5	0 (<1)	2,200 (>99)	28 (1.3)
P6	87 (5.7)	1,400 (92)	28 (1.9)
M1	0 (<1)	10,200 (>99)	37 (<1)
M2	0 (<1)	4,400 (99)	55 (1.3)
M4	2,800 (6.4)	41,000 (93)	115 (<1)
C1	0 (<1)	5,100 (97)	170 (3.3)
C2	24 (<1)	7,800 (>99)	47 (<1)
C3	0 (<1)	3,800 (>99)	15 (<1)
C5	145 (<1)	45,200 (>99)	86 (<1)

Partially purified hCG was applied to a column of DEAE-Sepharose (see purification scheme, Fig. 1) and was eluted stepwise with 0.02 M Tris-HCl, pH 8.0, and the same buffer plus 0.15 and 0.20 M NaCl. The column was stripped with 1.0 M NaCl and equilibrated with 0.02 M Tris-HCl, pH 8.0, before reuse. Values are the total and proportion of hCG immunoreactivity (Hybritech Tandem-R test) eluting with each buffer. The percentage of the total is given in parentheses.

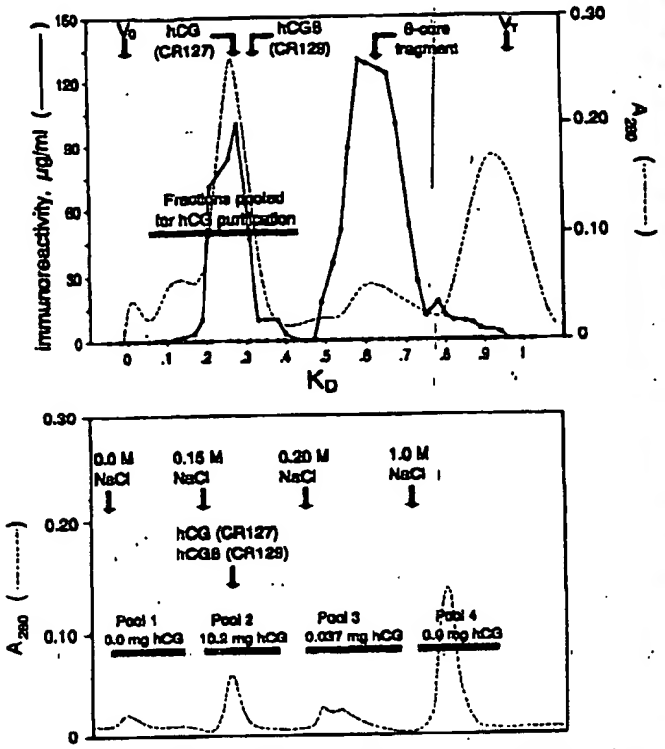

FIG. 2. Representative elution profiles for the first gel filtration (*upper panel*) and DEAE-Sepharose (*lower panel*) purification steps. The example is hCG extract M1 (see Table 1 and Fig. 1 for details). *Arrows* indicate elution positions of standards [hCG batch CR127, hCGβ batch CR129, hCG β-core fragment; blue dextran = void volume (V_0) and vitamin B_{12} = total volume (V_T)] and buffer change positions for DEAE-Sepharose chromatography. hCG levels were determined using the Hybritech Tandem assay (●). β-Core fragment immunoreactivity (○) was determined as described previously (10). The protein concentration is indicated by absorbance at 280 nm (- - -).

Other methods, such as amino acid analysis (Table 3), N-terminal sequence analysis (Table 4), and immunoassays (Table 5) were also used to evaluate purity. Eight of the 13 preparations (P1, P3, P5, M1, M2, M4, C3, and C5) had amino acid analyses similar to the hCG reference preparation CR1 19 (Table 3) and had only hCG α- and β-subunit-related N-terminal sequences (Table 4). These eight preparations were considered pure. Two other preparations (C1 and C2) had amino acid analyses inconsistent with standard hCG, although they only had hCG α- and β-subunit-related N-terminal sequences. Immunoassay data (Table 5) indicated the presence of free β-subunit in these two samples. These two preparations were considered hCG contaminated with free β-subunit. The remaining three preparations had irregular amino acid analyses and N-terminal sequence data consistent with the presence of hCG and a small amount of contaminant protein. The contaminant protein starts with the sequence NH_2Lys-Glu-X-X-Ala-Lys-Lys-Phe-Gln-,

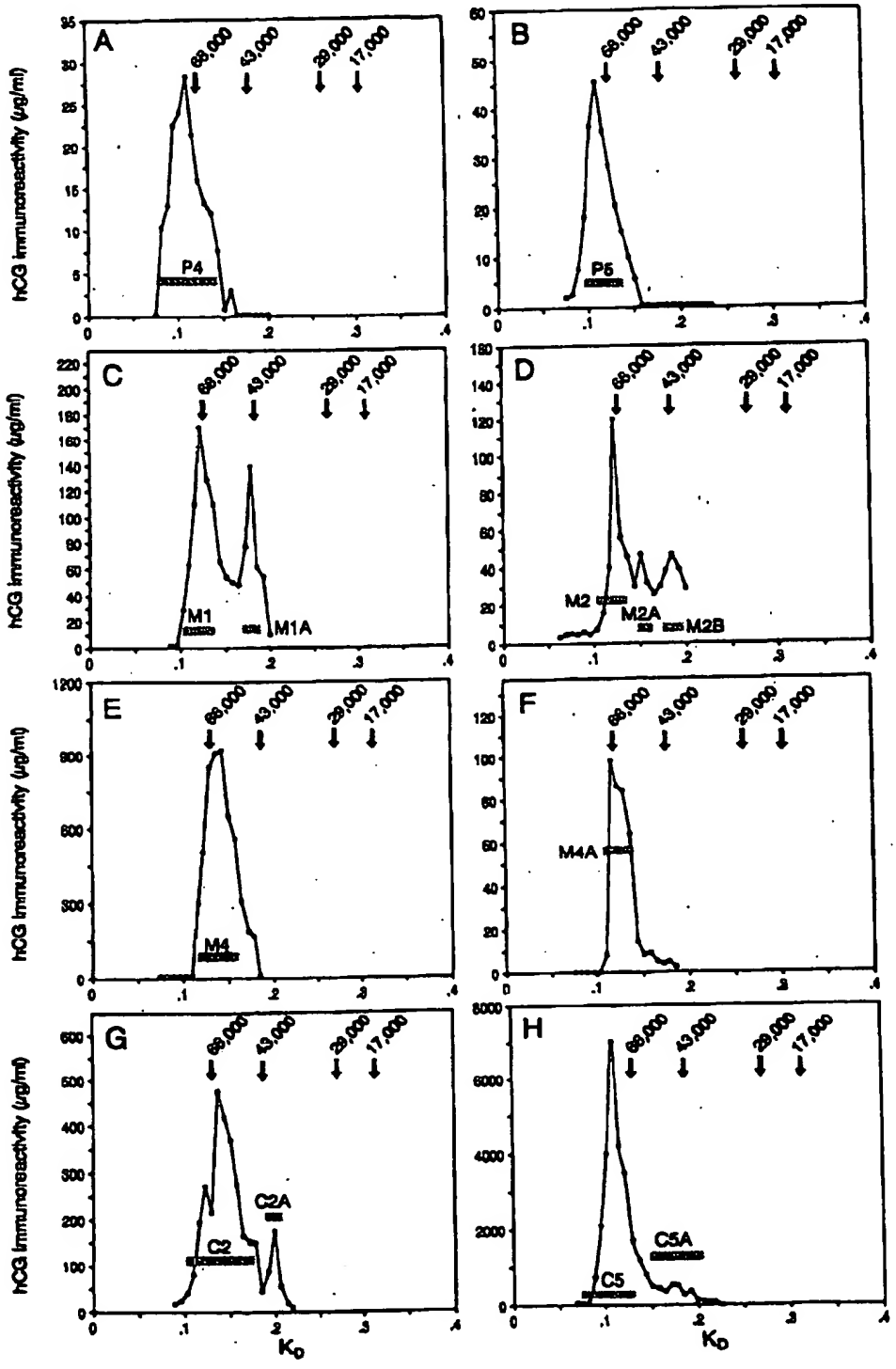

FIG. 3. Gel filtration on Sephacryl S200 (high resolution) of partially purified hCG preparations (after DEAE-Sepharose chromatography). A and B are preparations from pregnant women P4 and P5; C, D, and E are preparations from individuals M1, M2, and M4 with hydatidiform mole; G and H are preparations from individuals C2 and C5 with choriocarcinoma. F is the unbound fraction on DEAE-Sepharose from the preparation from individual M4. Samples (<5 ml) were applied to the first of two columns (each 2.6 × 90 cm) in series. Fractions were collected and assayed for hCG using the Hybritech Tandem-R kit. The *ordinate* is hCG in micrograms per ml; the *abscissa* is the gel filtration distribution constant, or elution volume, as relates to the column separation range (void volume = 0; total volume = 1.0). *Arrows* show distribution constants and M_r of gel filtration standards, BSA, ovalbumin, carbonic anhydrase, and myoglobin, respectively. *Shaded areas* indicate fractions pooled.

which does not correspond to any human sequence listed in the PIR peptide database.

Additional peaks of hCG immunoreactivity were found at the DEAE-Sepharose and second gel filtration purification steps. These indicated the charge and size heterogeneity of hCG. As shown in Table 2, a small unbound component was detected in samples P2, P6, and M4 on DEAE-Sepharose. This accounted for 2.6%, 5.7%, and 6.4%, respectively, of the total hCG immunoreactivity. There was a sufficient amount of M4 unbound material